Interferences in immunoassays

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Review

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Interferences in immunoassays: review and practical algorithm

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Abstract: Immunoassays are currently the methods of choice for the measurement of a large panel of complex and heterogenous molecules owing to full automation, short turnaround time, high specificity and sensitivity. Despite remarkable performances, immunoassays are prone to several types of interferences that may lead to harmful consequences for the patient (e.g., prescription of an inadequate treatment, delayed diagnosis, unnecessary invasive investigations). A systematic search is only performed for some interferences because of its impracticality in clinical laboratories as it would notably impact budget, turnaround time, and human resources. Therefore, a case-by-case approach is generally preferred when facing an aberrant result. Hereby, we review the current knowledge on immunoassay interferences and present an algorithm for interference workup in clinical laboratories, from suspecting their presence to using the appropriate tests to identify them. We propose an approach to rationalize the attitude of laboratory specialists when faced with a potential interference and emphasize the importance of their collaboration with clinicians and manufacturers to ensure future improvements.

Keywords: algorithm; immunoassay; interference; patient care.

Introduction

Immunoassays are currently the methods of choice for the measurement of a large panel of complex and heterogenous molecules owing to full automation, short turnaround time (TAT), high specificity, and sensitivity [1, 2]. This is achieved by using antibodies capable of binding antigens with high specificity and affinity. Nowadays, most immunoassays performed in laboratories are automated, based on competitive (i.e., limited reagent assays) or non-competitive (i.e., excess reagent, two-site or sandwich assays) formats, and involve various types of labels for signal detection (e.g., enzyme-linked immunosorbent assay, enzyme multiplied immunoassay technique, electrochemiluminescence immunoassay, luminescent oxygen channeling assay) [3]. In competitive assays, the signal is inversely proportional to the antigen concentrations while the signal is directly related to the antigen concentration in two-site assays. Various immobilization systems for reagent antibodies are also used, with the predominant one being the biotin-streptavidin interaction. These techniques are however prone to several types of interferences that may lead to harmful consequences for the patient (e.g., prescription of an inadequate treatment, delayed diagnosis, unnecessary invasive investigations) [1, 4–7]. In recent reviews, it has been estimated that at least 45–50% of documented interferences in cardiac or thyroid assays led to misdiagnosis and/or inappropriate treatment [6, 8]. Interferences exhibit various characteristics: their concentration may fluctuate with time, they may cause either false negative or false positive results depending on their nature, they are unique to an individual and are often specific to an analytical method [9–12]. Currently, the frequency reported for interferences in immunoassays ranges from 0.4% to 4.0% [13]. The identification of an interference remains a challenging process that may be improved and facilitated by a thorough step-by-step approach. The aim of this review is to recapitulate the current knowledge on interferences in immunoassays and to propose a practical algorithm to help laboratory professionals and clinicians to identify interferences and avoid wrong interpretation of laboratory results. We will discuss this algorithm by going through all major concepts, from suspicion of an interference to relevant tests to perform.

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Algorithm

Suspicion of an interference

Although systematic investigation of interferences in each sample would increase their detection, such an approach is impractical in clinical laboratories as it would notably impact budget, TAT, and human resources [1, 14, 15]. However, under specific conditions, a systematic search for interferences could prove cost-effective [4] (*see chapter 3*). Some strategies focusing on exploitation of patient data have also been proposed to increase their detection (e.g., Bayesian statistics, patient median follow-up) [13, 16]. Such approaches should however be considered as a starting point and need further validation [4, 15]. In the absence of better recommendations, the identification of an interference is nowadays mostly performed on a case-by-case basis. Hereby, we propose a detailed algorithm that aims at handling suspected cases of interferences (Figure 1). The first important step of the algorithm is the suspicion of an interference. A discordance between results, clinical presentation, clinical and biological history and/or with other biological measurands is suggestive of an interference [1, 2, 6, 7]. Clinicians are at the frontline to detect inconsistencies between laboratory results and clinical presentation, and to consistently notify these findings to the laboratory. Their role in pre-preanalytical steps (i.e., tests ordering) is also decisive as they are in possession of valuable medical data on each of their patients and may be able to communicate relevant elements through their requests to the laboratory. Laboratory specialists and staff on the other hand are able to prevent some preanalytical errors and to identify incoherent results when comparing them to previous results or to other measurands (e.g., relations between thyroid stimulating hormone (TSH) and free thyroxine (FT4) and/or free triiodothyronine (FT3), alkaline phosphatase (ALP), calcium and 25-hydroxyvitamin D (VitD); androstenedione, dehydroepiandrosterone sulfate (DHEA-S) and testosterone; cardiac troponin (cTn), creatinine-kinase (CK) and CK-myocardial band (CK-MB)) [4, 13, 14, 17]. The use of appropriate reference intervals (e.g., age, sex) and contextualization (e.g., medication, fasting state, posture (e.g., renin), physical activity (e.g., increase in D-dimers, N-terminal pro-brain natriuretic peptide (NT-proBNP), cTn in elite athletes) [18, 19], circadian cycle (e.g., cortisol, adrenocorticotropic hormone (ACTH), testosterone, TSH, stress (e.g., prolactin (PRL), growth hormone (GH), thyroid hormones) are major elements to take into consideration before suspecting an interference [14, 19–21].

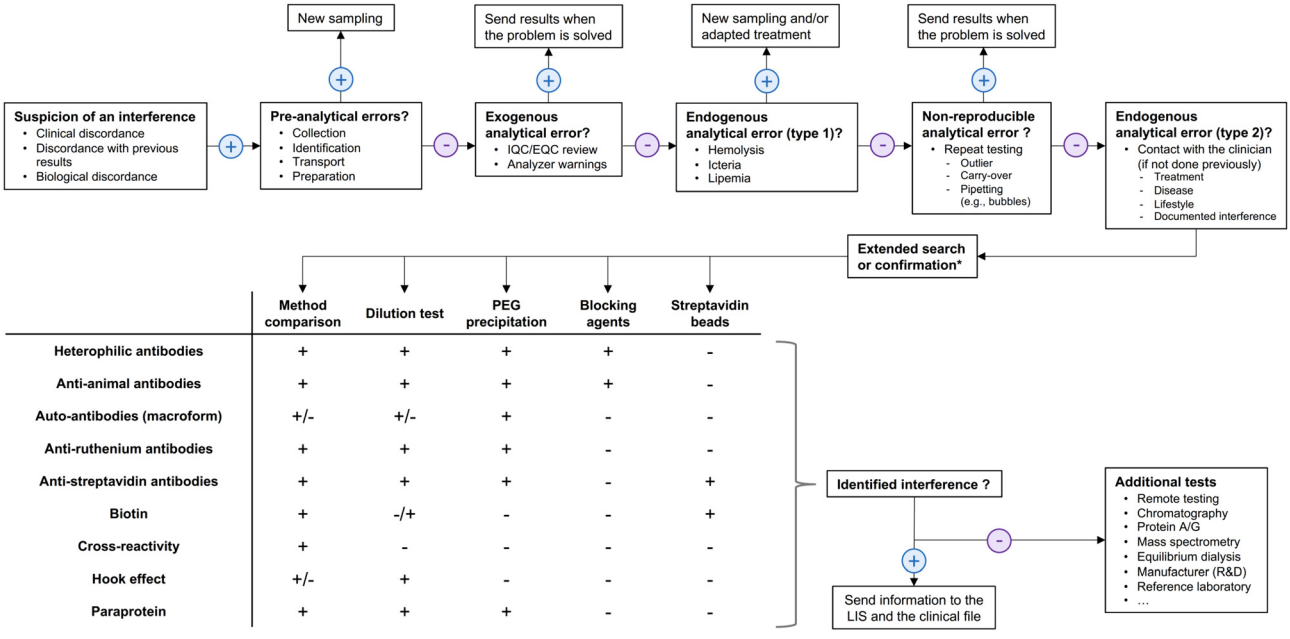

Figure 1: Practical algorithm for interference investigation in immunoassays.
EQC, external quality control; IQC, internal quality control; LIS, laboratory information system; PEG, polyethylene glycol. *Depending on the information obtained from the clinician, a particular test could be performed in priority (e.g., method comparison or streptavidin beads treatment in case of biotin medication).

Exclude a preanalytical error

Preanalytical errors are the most encountered errors in laboratories [19, 22]. They are usually divided into four categories: (1) sampling errors (e.g., veinous stasis, order of blood tubes draws, anticoagulant type, tube filling, homogenization), (2) identification errors, (3) transportation errors (e.g., stability, temperature), and (4) preparation errors (e.g., centrifugation, aliquoting) [22, 23]. Among these, the main preanalytical errors are: incorrect samples identification, invalid tubes filling, inadequate choice of tubes, clotted samples, inaccurate analysis request form, faulty transportation and conservation, and *in vitro* hemolysis [19]. These preanalytical errors will potentially have a harmful effect on patient care and are associated with additional costs [24]. Such errors should therefore be excluded before carrying on with the search for another source of interference. As such, identification of the patient should be confirmed [1, 7], tube integrity should be inspected (e.g., additives) [12], and compliance with good laboratory practices should also be verified (e.g., tube type, tube filling, clotting) [25]. The adequate filling of the tube is mostly important for analyses measured on tubes that use citrate as an anticoagulant (e.g., D-dimers) and should be respected to avoid falsely negative results [26]. Although most measurands are stable at room temperature for several hours, some of them are sensitive to transportation conditions. It is for example recommended to assay ACTH on a tube using ethylenediamine tetraacetic acid (EDTA) (a calcium chelator to limit enzymatic degradation) anticoagulation [2, 12]. EDTA and citrate may however interfere with some assays through chelation of europium labels [14]. Lithium heparin tubes are generally accepted in hormone quantitative analysis [27–29]. Fibrin can be generated from residual fibrinogen in case of inadequate post-phlebotomy tube homogenization and may cause interferences [27–29]. Inadequate mixing can also lead to decreased stability in sensitive measurands [12]. Serum samples on the other hand are not affected by anticoagulants as they do not require any and exhibit a good stability for the majority of biological measurands [14]. However, this type of tube needs a clotting phase of 30 min to 1 h before centrifugation to eliminate fibrinogen, fibrin and blood cells that may interfere with the analysis, therefore prolonging the global TAT [28]. Some measurands such as parathyroid hormone (PTH) deteriorate faster in serum than in EDTA [14, 30]. Renin, glucagon, and gastrin also are delicate measurands [2, 12, 14]. Moreover, the use of separating gel can influence the analysis of some measurands that may adsorb on it (e.g., phenobarbital, phenytoin, carbamazepine (few hours) and progesterone (few days)) [14]. Finally, time of collection and transportation conditions from collection location to the laboratory are crucial elements to determine as they can impact measurand stability [31]. If such a preanalytical error is detected prior to the analytical phase, a new sample is required and should be monitored to ensure adequate realization of the analyses [1].

Exogenous vs. endogenous analytical errors

Analytical errors are commonly classified as exogenous errors or endogenous errors [1]. Exogenous errors are to be linked to analytical procedure impairments (e.g., calibrator or reagents degradation, imprecise pipetting, washing issues). These should be excluded through an adequate quality monitoring by checking results from internal (IQC) and external quality controls (EQC) and through a careful analysis of automatically issued messages from the analyzer. Then, endogenous errors are subdivided in two categories: type 1 endogenous errors (i.e. hemolysis, icteria or lipemia (HIL)) that can be detected prior to the analytical phase and type 2 endogenous errors (e.g., heterophilic antibodies, biotin, autoantibodies) that are hardly detected during the preanalytical phase [7]. The latter are not detected by a thorough follow-up of IQC and EQC [4, 13]. The various exogenous and endogenous errors that can be met in immunoassays are depicted in Table 1. Although type 1 endogenous errors are progressively better detected by modern analyzers using spectrophotometric measures, their existence should still be considered, even if immunoassays are generally less impacted compared to photometric assays [2, 7, 10]. Adequate thresholds can be applied to detect lipemia interference and different methods can be pursued to overcome the erroneous result (e.g., ultracentrifugation, high-speed centrifugation, lipid-clearing agents) [32–34]. If a type 1 endogenous error is detected and cannot be eliminated, a new sample is required.

Repeat testing and contact with the clinician

Before reaching the clinician regarding an aberrant result, a re-run analysis under the exact same conditions could permit to exclude a non-reproducible result (e.g., carryover, microparticles, fibrin, undetected air bubbles) [1, 6]. Such non-reproducible errors have been reported for several measurands including cTn and were shown to vary across analyzers [35–40]. Of note, these non-reproducible errors could rarely be detected through a thorough monitoring of quality controls or by the analyzer.

After exclusion of preanalytical, exogenous, type 1 endogenous and non-reproducible analytical errors, a contact with the clinician is essential to go further. Indeed,

Table 1: Exogenous and endogenous analytical errors in immunoassays.

Exogenous errors	
– Incorrect or degraded calibrator	
– Incorrect or degraded reagent	
– Reagent lots variation	
– Incorrect control reconstitution	
– Pipetting problems	
– Inadequate washing	
– Inadequate temperature	
– Undetected bubbles	
– ...	
Endogenous errors	
Type 1	**Type 2**
– Hemolysis	– Heterophilic antibodies (including rheumatoid factor)
– Icteria	– Anti-animal antibodies (e.g., HAMA)
– Lipemia	– Auto-antibodies (macro-complexes)
	– Antibodies against revelation systems (e.g., anti-ruthenium antibodies)
	– Immobilization system interferences (e.g., biotin, anti-streptavidin antibodies)
	– Cross-reactivity (e.g., drugs, diseases, lifestyle)
	– Hook effect
	– Paraproteins

HAMA, human anti-mouse antibody.

relevant elements in the patient medical records can be very useful to identify type 2 endogenous interferences and determine the nature of interference:

- Crossed-reactivity: steroidal structures (e.g., prednisolone, spironolactone, fludrocortisone [2, 13], addictions (e.g., amphetamines, nasal decongestants) [19], renal insufficiency (e.g., c-terminal PTH fragment accumulation, presence of 5-alpha-tetrahydroxycortisol, carbamazepine metabolites) [2, 19], children (e.g., VitD C3-epimers accumulation) [5, 41], antidote (e.g., digoxin immune Fab) [12, 19].
- Immunization or autoimmune diseases (anti-animal antibodies, heterophilic antibodies, autoantibodies): treatment using monoclonal antibodies, contact with animals (e.g., veterinarian, farmer, lab staff), viral or bacterial infection, transfusion, chronic allergy [13], vaccination [14], rheumatoid factor-positive autoimmune disease [1, 14, 19], disseminated erythematous lupus, rheumatoid arthritis, Hashimoto thyroiditis, Graves-Basedow disease [6], etc.
- Hook effect and paraprotein: recent diagnostic or follow-up in oncology [19].
- Biotin: food supplement intake, metabolic disease, multiple sclerosis [42, 43].
- Carrier protein: pregnant women, contraceptive pill [2], antiepileptic [14], diabetic ketoacidosis or heparin administration (accumulation of free fatty acids that modify binding of thyroxin with its carrier protein that can give an artefactual elevation of FT4) [2, 6].
- Others: contrast agent [44], hemoglobin-based oxygen carrier [45], etc.
- Interferences referenced in the patient's medical record [7]. Of note, an interference known in a mother can be found in her newborn.

If no such interference can be suspected based on medical history or if confirmation is needed, additional tests are ultimately required for interference investigation.

Extended search or confirmation

To identify type 2 endogenous errors, two scenarios are generally proposed. The first consists of using the same sequence of complementary tests and the second considers the profile of the interference (i.e., probabilistic method). The data obtained from the discussion with the clinician can be used to drive the appropriate strategy (e.g., biotin intake, prednisolone treatment, contact with animals, previously described as macroTSH). It is important to remember that a single test is rarely sufficient to identify an interference [1, 6, 13]. It is therefore recommended to have several tests available to increase the chances of identifying interferences [1, 2, 6, 7]. The use of control patients is recommended when looking for interferences to determine whether it is reasonable to consider that the observed deviation is due to an interference or not [1, 13]. Most of the additional tests are simple to perform, require little volume and can be performed on the original sample. This prevents the need for an additional phlebotomy. However, if the left-over volume is too small, the clinician should be contacted to consider obtaining a new sample.

Method comparison

Method comparison is often presented as the first investigative step to consider [1, 2, 6, 7, 10, 12, 13, 19, 32]. Interferences are generally limited to certain analytical methods [11]. Therefore, the doubtful result should be compared with a different method (e.g., different antibody immobilization system (e.g., biotin-streptavidin), number of washes, contact between patient sample and tracer (e.g., one-step vs. two-step) [6], different type of antibody (e.g., mouse, sheep, horse), different detection systems (e.g., ruthenium, ALP

[12, 46])). If the method has been carefully selected, it will be possible to detect most interferences. Competitive immunoassays are the most affected by cross-reaction caused by a lack of antibody specificity, which will lead to false positive results [11, 12]. Data on possible cross-reactions for certain drugs or hormones are available in the literature and manufacturers of immunoassays normally provide such information in insert sheets [13]. Over time, improvements have been made in reducing cross-reactions [2, 19]. For example, the reactivity of prednisolone in cortisol immunoassays performed on Roche Diagnostics (Basel, Switzerland) platforms has decreased from 171% to 8% [2]. A recent review of the literature examined the impact of several drugs and metabolites on immunoassays [19]. Therapeutic drug and immunosuppressor monitoring immunoassays are often and mainly prone to cross-reactivity caused notably by drug metabolites or endogenous compounds, potentially causing major impacts on patient care (i.e., mainly through falsely elevated drug concentration). Heterophilic antibodies, HIL or paraproteins may also interfere in some assays. Although immunoassays are widely used owing to shorter TAT and lower initial cost, liquid chromatography coupled to (tandem) mass spectrometry remains a gold-standard for therapeutic drug and immunosuppressor monitoring because virtually unaffected by interferences [19].

If ruthenium antibody or biotin interference are suspected when using a method involving ruthenium as a detection system or biotin in its immobilization system, it is recommended to choose another method with different technical characteristics [6, 47, 48]. The average bias between the two analyzers used for comparison should be known for a correct interpretation about the possible presence of an interference [13]. If possible, a reference method such as equilibrium dialysis (e.g., FT4, testosterone) [49]) or mass spectrometry should be preferred (e.g., steroids, therapeutic monitoring, toxicants) [1, 5, 6, 19, 41, 49]. Paraprotein interference is usually method-specific and can therefore also be identified by comparing the doubtful result with another method [50–55].

A significant difference in results between two platforms generally confirms the presence of interference [13] but does not directly indicate the type of interference involved. Additional tests should therefore be used. Furthermore, method comparison is not always effective in identifying autoantibodies (or macrocomplexes) [11, 13, 56, 57]. This technique often requires sending a fraction of the sample to another laboratory [13].

Dilution test

In the absence of interference, the concentration of a measurand decreases progressively in a linear fashion as the sample is diluted. When interference is present, a loss of linearity is frequently observed as the sample is diluted [2]. Dilutions of 1/2, 1/4, and 1/8 are classically performed [13] and the manufacturer's diluent should be used whenever possible [1, 10]. The linear aspect should not be assessed visually but in a more objective way, including the coefficient of variation of the instrument for the measurand considered [13]. Testing control patient samples in parallel is also recommended. The dilution test is mostly useful for identifying interference from heterophilic antibodies, anti-animal antibodies [13], anti-ruthenium antibodies [47], anti-streptavidin antibodies [58, 59], and paraproteins [50, 55, 60].

This test is quite simple and fast to use but has some disadvantages. Linear results after successive dilutions do not necessarily indicate the absence of interference [6,13,61], unlike what was suggested in the algorithm proposed by Lauro et al. [62]. Ismail et al. showed that up to 40% of samples with known interfering antibodies showed good linearity following the dilution test [9]. A loss of linearity may also be specific to certain methods [13, 59]. In addition, this dilution test is not very effective in identifying interference due to the presence of macroforms [6] or when cross-reactivity is suspected. The dilution test to identify biotin was only scarcely studied and more optimal solutions exist [63–65]. Moreover, free hormones (e.g., FT4, FT3) cannot be diluted, except under specific validated conditions, because dilution causes a disruption in the equilibrium with the related carrier proteins [66].

The dilution test is however the preferred one to identify hook effect, usually using progressive dilutions (i.e., 1/100, 1/1,000) [19]. The hook effect affects measurands that may present very high concentrations (e.g., alpha-fetoprotein, carcinoembryonic antigen, cancer antigen 125, prostate specific antigen, β-human chorionic gonadotropin (hCG), kappa/lambda free light chains, PRL, Tg, ferritin, GH, urine albumin) and/or when the amount of antibodies used in the kits is low [2, 19, 32, 49, 67]. The hook effect occurs almost exclusively in one-step "sandwich" formats (i.e., capture and detection antibodies added at the same time) [12]. The measurand in excess saturates the capture and detection antibodies, preventing the "sandwich" from forming [49]. During rinsing, only a limited amount of "sandwiches" is formed and the signal produced is weaker than it should be, resulting in low or medium high concentrations [49]. Suppliers provide threshold concentrations below which no hook effect is documented. Incubating the sample with the capture antibody first and adding the detection antibody after a washing step also prevents hook effect [49].

PEG precipitation

Polyethylene glycol (PEG) 6000 acts somewhat like a sponge, by trapping water present in protein structures, thus altering their solubility and causing them to precipitate [56]. Higher molecular weight proteins have lower solubility compared to lower molecular weight proteins [2]. Sample treatment with PEG 6000 (25% w/w) is classically performed to identify the presence of autoantibodies (macroforms) but is generally useful to determine whether an antibody is responsible for the presumed interference (e.g. anti-streptavidin antibodies [58, 59], anti-ruthenium antibodies [47], or paraprotein [55, 60]). PEG will therefore be useless to identify biotin interference [6], interferences due to a lack of specificity (e.g., spironolactone, prednisolone) or hook effect (e.g., tumor markers in high concentrations).

Macroprolactin (macroPRL) is a well-known macroform and is a complex formed between PRL and an immunoglobulin (mainly immunoglobulin G (IgG)). This complex is considered inactive, shows a lower clearance, and can therefore accumulate in the circulation [19, 49]. In normal subjects, the proportions of monomeric, dimeric (or big PRL) and macroPRL (or big-big PRL) forms are about 86%, 9%, and 5%, respectively. Macroprolactinemia is defined as a predominant level of macroprolactin in the serum of an individual [49]. The antibodies used in the kits currently found on the market are not able to distinguish between the monomeric form of PRL and macroPRL. False positive results are therefore observed. The prevalence of macroPRL in the general population is about 4%. In patients with hyperprolactinemia, the prevalence varies between 4% and 46% of cases [68, 69] and marked geographical variation has been recently reported [70].

Other macroforms have also been documented: macrocobalamin (macroB12) (18% of cases of cobalamin (B12) >1,476 pmol/L) [57], macroTSH (0.6% to 1.6% of increased TSH cases) [6], or macro-cardiac troponin (macrocTn) (5% of cases of increased cTn [71], affecting more frequently cTnI than cTnT [72]). Although some platforms are less sensitive to the presence of certain macroforms, none is 100% insensitive [6, 19, 56, 57]. Comparison of methods will therefore not always be useful. It is recommended to use post-PEG reference values adapted to the measurand in question [1, 6, 57]. These references values are to be determined by the laboratory professionals or can be found in the literature [57, 68, 73]. The calculation of a recovery percentage (pre-PEG result/post-PEG result; %) is no more recommended since it may mask the presence of a macroform associated with a real excess of monomeric forms [6, 57]. Co-precipitation of monomeric forms may be increased when high levels of globulins are encountered, leading to erroneous conclusion of macroprolactinemia [56]. PEG may also interfere with some assay platforms (e.g., Immulite (Siemens Healthineers, Erlangen, Germany), Access (Beckman Coulter Inc., Brea, USA)). PEG precipitation of immunoglobulin A (IgA) is suboptimal and a macroprolactin composed of IgA could therefore be missed [56]. It is therefore recommended to confirm the presence of a macroform by using gel exclusion chromatography (GEC) [1, 6]. One study also showed that PEG precipitation interfered with the quantification of certain antibiotics [74]. For obvious reasons, PEG cannot be used when assays target serum antibodies (e.g., anti-thyroperoxidase (TPO), anti-thyroglobulin (Tg)) [13].

Recently, the possibility of automating PEG processing has been demonstrated [75]. This kind of approach, together with the availability of fast-track procedures [76], and post-PEG reference values [57, 73] support the widespread use of macroform searching.

Blocking agents

Heterophilic antibodies are low-affinity poly-specific antibodies formed early in the immune response, directed against poorly defined epitopes. This definition notably includes rheumatoid factor. Anti-animal antibodies are monospecific, high-affinity antibodies directed against defined animal epitopes (horse, goat, sheep, and more frequently mouse) [2, 77–80]. The term "heterophilic antibodies" should therefore be used when there is no evidence of exposure to an animal antigen (e.g., monoclonal antibody treatment, close contact with animals). Only high-titer antibodies with high affinity for the assay antibodies will cause erroneous results and possible harmful clinical consequences [19].

The type of interference produced is the same for heterophilic and anti-animal antibodies. False positive results in a sandwich assay format are more frequently reported where the interfering antibody acts as a bridge between the capture and detection antibodies [6, 10, 19, 32, 80]. The incidence of heterophilic antibodies and anti-animal antibodies has been estimated to range from 0.05% to 6%, depending on measurands and studies considered [10, 81, 82]. Low concentrations of rheumatoid factor are present in about 5% of the population, and in about 70% of patients with rheumatoid arthritis [14].

The presence of these interfering antibodies has been reported for a wide range of measurands (e.g., cTn, TSH, insulin, D-dimer, AFP, BNP, hCG, GH, cyclosporine, tacrolimus, human immunodeficiency virus) [6, 19, 83, 84]. Aware of the interference related to heterophilic antibodies

and anti-animal antibodies, manufacturers try to limit their occurrence by adding blocking agents to reagent kits. Choosing Fab or F $(ab')_2$ fragments instead of intact immunoglobins for the selection of capture and detection antibodies also eliminates interference specific to the Fc portions of antibodies. Choosing chimeric antibodies with an animal variable portion is yet another solution [12]. However, this is not always effective [1, 12]. Blocking agents are a simple solution to demonstrate such interferences. It is preferred to use non-immune serum due to the better detection rate described [13]. Blocking agents are composed of an immunoglobulin cocktail that can neutralize endogenous antibodies [13]. Several commercial solutions exist (e.g., heterophilic blocking tubes (HBT) (Scantibodies Laboratory Inc., Santee, USA), HeteroBlock (Omega Biologicals Inc., Bozeman, USA), MAB33 (monoclonal mouse IgG1)) [19]. A significant change after treatment indicates the presence of interference [13]. An absence of change after a blocking treatment is however observed in 20–30% of interference cases [1, 2]. Increasing the concentration of blocking agents with the same amount of sample can in some cases identify a high-titer interference [1, 6]. As stated by the manufacturer, HBT perform less well against human anti-mouse antibody (HAMA) interference [2]. Although two papers have shown that interferences due to anti-streptavidin antibodies [59] and paraprotein [85] have responded to treatment with blocking agents, the latter are mostly useless to demonstrate the presence of heterophilic antibodies or anti-animal antibodies.

Neutralization of biotin/anti-streptavidin antibodies

Biotin (also known as vitamin B7 or B8) is an essential coenzyme involved in fat, protein, and carbohydrate metabolism [42]. Biotin is used, together with streptavidin or avidin, as an immobilization system in many immunoassays [86, 87]. Low concentrations corresponding to a daily dose of 30–35 µg cause no interference with immunoassays. Higher doses of biotin used for cosmetic purposes (e.g., hair, nails, skin; 5–10 mg), or for therapeutic purposes (e.g., metabolic disease, certain forms of multiple sclerosis; up to 300 mg/day) may, however, cause interferences [42]. In a sandwich immunoassay, false negative results are encountered (e.g., TSH, cTn, PSA, hepatitis B surface antibody) whereas competitive immunoassays are affected by false positive. results (e.g., FT4, testosterone, total hepatitis B core antibody, hepatitis B antibody) [6, 88, 89]. It was shown that the percentage of American whose biotin intake was at least 1 mg/day increased from 0.09% in 1999 to 2.6% in 2016 [90]. Another study showed that between 15 and 20% of Americans reported using biotin supplements [43]. Furthermore, it was observed that 7.4% of patients admitted to the emergency department in America presented a biotin concentration high enough to cause interference (≥10 µg/L) [91]. Importantly, it is known that about 60% of immunoassays performed in France and the USA use biotin in their immobilization system [86, 87]. This may therefore be a concern for laboratories using biotin-susceptible methods. However, a study on 572 cardiac intensive care unit patients showed that the concentration of biotin measured was not sufficient to interfere with the cTn assay [92], while others reported that biotin intake showed little likelihood to produce false positive results on cTnT assay on patients with suspected acute myocardial infarction. In these patients, the 99th percentile biotin concentration was 2.63 µg/L [93]. Another study that included 1,487 samples from pregnant women showed that only 3% had a biotin concentration greater than 1.0 µg/L with a maximum measured concentration well below the interference threshold reported by Roche (70 µg/L) [94].

Several methods exist to identify/neutralize this interference:

- Consultation of the medical record of the patient or ask the patient. This method can only identify few cases because the intake of food supplements with high doses of biotin are generally not reported by the patient.
- Determination of biotin concentration by microbiological methods, enzyme-linked immunoassay, liquid chromatography tandem mass spectrometry or rapid qualitative solutions (e.g., VeraTest Biotin (Veravas, Austin, USA)) [65, 95]. However, these methods are not readily available and can be quite expensive. In addition, the lack of standardization between them does not allow the same thresholds at which interference should be suspected to be applied [65].
- Mixing the sample with streptavidin beads. Biotin will bind to streptavidin on the beads in excess. A new analysis for the measurand after removing the beads by centrifugation or with a magnet will therefore overcome the interference of biotin. The impact of these treatments on the measurands to be assayed is quite low (i.e., mostly <10%). These beads can be recycled from supplier kits [96] or purchased as ready-to-use solutions (e.g., VeraPrep Biotin, (Veravas, Austin, USA)) [65]. This method quickly identifies biotin interference [97].
- Waiting for the patient to eliminate biotin by renal pathways before performing the test. A 24 h period is often sufficient to eliminate biotin. However, some patients with renal failure may require a longer washout period. This also implies a delay in patient

management and the need to interrupt biotin intake for several days (not desirable for the treatment of metabolic disease for example) [42, 65].

– Assaying the sample using a biotin insensitive method (e.g., architect (Abbott, Abbot Park, USA)). Roche Diagnostics is currently working on a new generation of immunoassays that are more resistant to biotin [42]. Initial results for cTn and TSH showed no interference up to biotin concentrations of 1,200 µg/L [98]. The new generation of Tg assay was also shown as unaffected by biotin up to 1,560 µg/L [99]. In Figure 2, we performed a spiking experiment with biotin on the previous and new generation of NT-proBNP assays from Roche Diagnostics (Figure 2). Five patients were included with different NT-proBNP concentrations and 5 concentrations of biotin have been spiked (91, 495, 824, 1,200 and 3,500 µg/L) based on the procedure presented by Trambas et al. [100]. The new generation assay was insensitive to the presence of biotin up to 3,500 µg/L. Using the previous generation, a significant NT-proBNP decrease was observed from 495 µg/L. Obviously, the use of insensitive assays represents the best solution to overcome biotin interference.

First described in 2013 [101], anti-streptavidin antibodies (immunoglobulin M (IgM) or IgG), cause interference similar to biotin: false negative results for a sandwich format and false positive results for a competitive format [102, 103]. Their prevalence has been estimated at 0.6% [102]. Most of the reported cases have been described for thyroid function tests [58, 59, 101, 104, 105] but interferences with other measurands have also been reported (e.g., cortisol, testosterone, DHEA-S, anti-Tg, anti-TPO) [58, 101]. Their origin remains unknown. The hypothesis of an immune reaction against the ubiquitous bacterium *Streptomyces avidinii* has been formulated [103]. Assay of the sample containing anti-streptavidin antibodies on an insensitive method or treatment with streptavidin beads are effective in overcoming the interference [58, 59]. Observing a washout period is not effective with antibodies, although their titer appears to decrease over time [6, 103]. Treatment with PEG 6000 has been shown to be effective in precipitating anti-streptavidin antibodies [58, 104, 106].

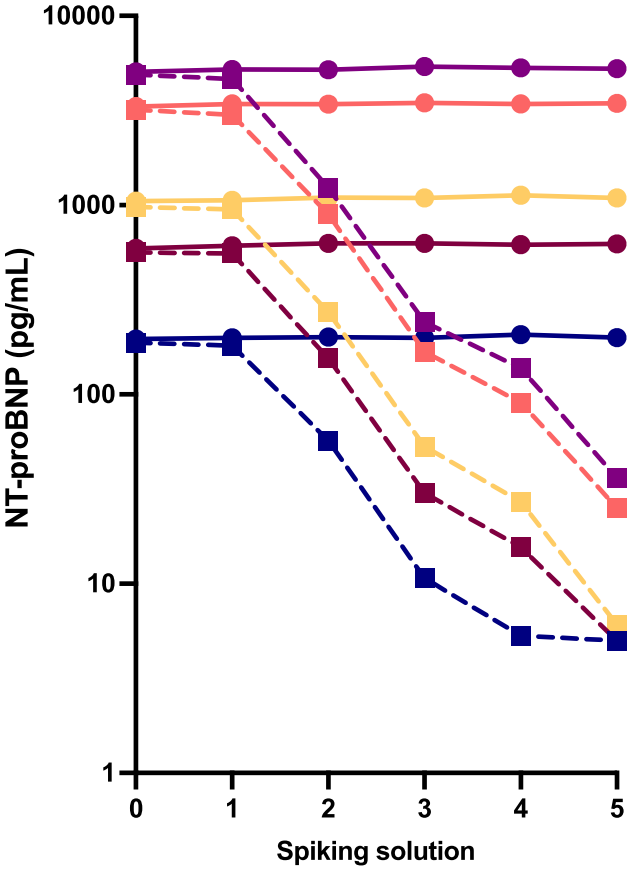

Figure 2: Effect of biotin spiking on previous and new generation Roche Elecsys NT-ProBNP assay.
Dotted lines correspond to the previous generation assay and solid lines to the new generation assay. Colors represent different patients. Spiking biotin concentration were as follow: 1 = 91 µg/L; 2 = 495 µg/L; 3 = 824 µg/L; 4 = 1,200 µg/L; 5 = 3,500 µg/L.
NT-proBNP, N-terminal pro-brain natriuretic peptide.

Additional tests

To complement the classical tests presented above, additional tests can also be used depending on the suspected interference. Lauro et al. propose to perform a new blood sampling when an inconsistent result is observed [62]. This will mostly avoid interference from biotin or other compounds with a relatively short half-life. However, some interfering antibodies may remain in the circulation for a long time. The production of interfering antibodies may also be chronic (exposure to animals, allergies, autoimmune diseases) [13]. Interferences lasting several years have indeed been reported [6, 107]. Some treatments are also taken on a long-term basis (e.g., heparin, biotin) and therapeutic discontinuation is not always feasible. If an interference has been reported in the past and the same interfering pattern is observed, it is likely that the interfering cause remains the same (e.g., antibodies, chronic treatment). In this case, and if applicable, the appropriate response is to apply the methodology that identified it in the past to confirm the nature of the interference. To confirm the presence of a paraprotein, protein electrophoresis and immunofixation can be used [2, 52, 85] or the interference can be precipitated in the presence of ethanol

[74]. In some cases, it is also possible to measure certain measurands in the urine to determine whether the result is consistent with the blood test. For example, if a hCG level is assumed to be erroneous on a blood sample, a negative urine assay could be useful to confirm an interference in the blood [1, 2]. This is because antibodies (e.g., heterophilic antibodies) do not pass into the urine [19]. Interfering antibodies can be effectively extracted by treatment with protein A or G. This method has superior specificity compared to PEG [6]. GEC also remains the reference method for macroform identification [2]. In some cases, however, it is difficult to distinguish between macroform interference and animal antibodies, as they exhibit a similar chromatographic pattern and are both sensitive to PEG treatment [2]. To distinguish between them, it was proposed to incubate the suspect sample with a control sample (1:1 ratio for 4 h) with a high concentration of measurand (e.g., high TSH). A low percentage of recovery after 4 h of incubation will be more in favor of a macroform [2]. Genetic analysis can also identify certain variants that cause changes in carrier protein binding (e.g., artifactual increase in FT4 [6]).

Once an interference is identified, it should be documented in the patient clinical record and in the laboratory's computer system to anticipate possible future interferences and thus enhance efficiency [7]. If no interference has been identified despite strong suspicion and several additional tests, the sample can be sent to a reference laboratory [1, 2]. It is also worth considering sending the sample to the manufacturer, who generally has the resources to perform more complex tests [47, 59].

Systematic search for interferences

As already mentioned, an extensive search for interferences in every patient sample is nearly impossible. However, in certain specific situations, systematic search should be considered. It is up to the laboratory, in partnership with clinicians, to determine whether such an approach would be cost-effective [1, 4]. This is particularly the case for the detection of type 1 endogenous errors, now well integrated on automated platforms [7]. The rejection of samples defined as non-compliant (e.g., wrong identification, wrong tube, wrong request) is therefore essential to avoid the occurrence of certain interferences [19]. Autoantibody investigation is also widely practiced. Given their high prevalence (i.e., 10% in the general population and up to 25% in cases of differentiated thyroid cancer), anti-Tg antibodies are frequently measured in association with Tg [1, 12]. The search for macroprolactinemia using PEG 6000 precipitation in any subject with hyperprolactinemia is also widely performed [7, 76]. Some proposed to look for macroTSH in case of TSH > 10 mU/L along with normal FT4 [108]. Recently, we have proposed to systematically search for the presence of macroB12 in case of a significant increase in B12 given its high prevalence (i.e., 18% in case of B12 > 1,476 pmol/L) [57]. The search for hook effect is also systematized for certain critical measurands by diluting the sample above a certain threshold [1].

As previously illustrated with biotin interference, *in vitro* diagnostics (IVD) companies are putting efforts to overcome common interferences. Identification and reporting of interferences in clinical laboratories and communication with manufacturers are crucial to encourage change. However, faced with technical limitations of immunoassays, a reasonable solution to foresee is using platforms that are less sensitive to certain types of interference, or even replacing immunoassay technology with mass spectrometry (e.g., steroid panel, therapeutic monitoring, toxicology screening), can reduce the occurrence of interference [1, 15, 49]. Future implementation of automated mass spectrometry could therefore be a game-changer in assay interferences [109]. Recent progresses in artificial intelligence are today a hot topic in laboratory medicine could also be part of the solution [110]. Zhou et al. recently published a delta-check method by using deep machine learning showing effective detection of sample mix-up identification. Such kind of innovations, if adapted to interference search, may potentially improve systematic approaches, and lay the foundations for automated interference workup.

Finally, raising the awareness of clinicians and laboratory professionals on this issue is essential as their expertise, along with the use of relevant procedures, may lead to effective interference detection.

Conclusions

The issue of immunoassay interferences is a critical topic in laboratory medicine since these assays are widespread and harmful consequences for the patient might be generated if not recognized. The nature of interferences is various, and their effects may differ across analytical methods. Systematic search is commonly performed for some interferences but remains challenging to implement in many situations. Therefore, a case-by-case approach should often be considered. To support it, we propose an algorithm based on current knowledge on immunoassay interferences. The expertise of laboratory professionals and clinicians is primordial to suspect an interference. This will trigger a sequence of steps including a careful review of possible

preanalytical, exogenous or type 1 endogenous analytical errors before performing extended investigations using a combination of tests for the identification of type 2 endogenous errors. Such a process remains time and manpower-consuming and would benefit from technical advancement and automation to ease its implementation in clinical laboratories. As such, this algorithm is proposed as support for field work and will hopefully serve as an impulse for further steps. Avoiding detrimental consequences on patient medical care through adequate management of interferences is crucial, and the collaboration between laboratory professionals, clinicians and IVD manufacturers is essential to ensure future improvements.

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